Granada Crystallisation Box: a new device for protein crystallisation by counter-diffusion techniques

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Granada Crystallisation Box (GCB) is a new crystallisation device designed to perform counter-diffusion experiments. Here we describe the device and its use for protein crystallisation purposes. GCB allows one to explore and exploit the coupling between crystallisation and diffusion. The role of viscous fluids, gels and/or microgravity can be enhanced by using capillary volumes, creating a perfect diffusive mass transport scenario. The use of capillaries also reduces the consumption of macromolecules and avoids the handling of crystals for X-ray diffraction data collection.

Keywords: **crystallisation device, protein, counter-diffusion**

1. Introduction

Biological macromolecules are currently crystallised from supersaturated or supercooled solutions (McPherson, 1999; Ducruix & Giegé, 1999). The simplest crystallisation method is the batch method in which the protein solution and the solution of the precipitating agent are mixed to achieve a supersaturated solution. The main recognised limitation of the batch technique is the lack of control over the development in time of supersaturation, because the supersaturation value is instantaneously achieved and the rate of change of supersaturation is fixed and controlled by the very growth process. This is a severe restriction for compounds nucleating at very high supersaturation (as is the case of most biological macromolecules), because the crystal grows (at least during the first stages) from highly supersaturated solutions required to attain nucleation, which limits the crystal quality and size. For this reason, most of the crystallisation protocols involving this kind of substances make use of some active mechanism to exceed supersaturation from an initially undersaturated or saturated solution. These mechanisms are based on temperature changes (thermal methods), evaporation of the solvent (vapour diffusion methods), or slow mixing of solutions either through a membrane (dialysis) or free liquid interface (free interface diffusion methods). These methods, when correctly adjusted, are able to produce crystals nucleated close to the lowest possible supersaturation. Three different problems are still present in these methods: first the rate of change of supersaturation must be adjusted for them to work, if the rate of increase of supersaturation is too slow, experimental time is wasted and, even worse, if it is too fast, the benefits of the method are lost due to the excess supersaturation accumulated during the nucleation induction time; second, this rate of increase of supersaturation is dependent on the solubility of the compound, so several screening experiments are needed to determine its optimum value; and third, very often the nucleation and growth kinetics are governed by more than one parameter, which severely increases the number of screening experiments required. To overcome these limitations, new methods have been proposed in which the nucleation and crystal growth proceeds far from equilibrium, the main practical consequence of this being the existence of large gradients of two or more of the quantities controlling supersaturation both across the system and along the experimental time. These gradients ensure that crystals growing at different points of the growth cell do it under a different supersaturation values and different rate of change of supersaturation, reducing the number (or even eliminating the need) of screening and rate optimisation experiments. This is the case of the so-called counter-diffusion methods (García-Ruiz, 1981; García-Ruiz, 2002).

Based on our experience in one-dimensional counter-diffusion methods (Henisch & García-Ruiz, 1985; García-Ruiz, 1991; García-Ruiz & Moreno, 1991; García-Ruiz *et al.*, 1993; Otálora & García-Ruiz, 1996) we have developed and made commercially available a new device for implementing counter-diffusion experiments (though not limited to them). The main features and uses of this device, called "Granada Crystallisation Box" (GCB), are described in this article. GCB is primarily intended for (though not limited to) the growth of crystals of biological macromolecules, so it will be described here in this context. The capabilities of GCB in other crystal growth experiments will be published separately. The advantages of this new setup, discussed through the text, can be summarized as follows:

(i) It works under diffusion-controlled mass transport, which is known to produce better ordered crystal lattices provided the growth proceeds in the diffusion controlled or mixed regime. The role of viscous fluids, gels or microgravity is enhanced by using capillary volumes

(ii) It automatically searches for the optimal crystallisation conditions. Because of the properties of counter-diffusion experiments, one single capillary scans the same precipitation region in the phase space as do many drops. As a consequence, only one capillary is needed per precipitating agent.

(iii) It reduces the consumption of macromolecule. Just 1 to 10 μl of protein solution is needed per capillary (smaller volumes can also be used for screening purposes in capillaries smaller that 0.1 mm in diameter). As the number of experiments required to get good crystals is reduced (see above), further reductions in global sample consumption are obtained.

(iv) It minimises the volume of the experimental set-up. 120 capillaries can be stacked in the holder shown in Figure 1. The design is modular and easily adaptable to different requirements in experiment storage. This is particularly useful in microgravity science. Up to 30 GCBs can be piled in a volume of about 10×10×10 cm.

(v) It is very well suited for X-ray analysis. GCB provides an easy and safe way to transport crystals to X-ray diffraction facilities. In situ X-ray crystallography is easy for crystals grown inside X-ray capillaries. No handling of crystals is required, eliminating the risk of crystal loss or quality degradation during handling and mounting. At the same time, GCB can also be used for cryo-crystallography, flash-cooling protocols have been tested successfully for GCB capillaries containing crystals as grown.

(vi) It makes easy the visualisation of the growth process. Transparent glass capillaries are very well suited for microscopic observation. The fact that they are contained themselves in a transparent box makes easier and safer the observation under the microscope.

2. Description of the GCB

Figure 1a shows a general overview of the GCB set including several boxes mounted on a rack. The GCB consist of three parts

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made of polystyrene: the box body (Figure 1b) consisting of a narrow box open on one side, the capillary holder (Figure 1c) that fits into the body and the cover lid (Figure 1b top) that closes the box. The box body has been designed to be small enough for experiments storage and transport yet being able to hold up to six different crystallisation experiments. The box is transparent and narrow to allow microscopic observation of the experiments. The rationale behind the design of the capillary holder is as follows: the density of experiments must be maximised but without compromising either easy insertion and handling of capillaries or the microscopic observation capabilities. The capillary holder can accommodate capillaries of diameter ranging from 0.1 mm to 1.5 mm. The construction of the holder as a separate part was decided for flexibility because this design allows the implementation of techniques other than counter-diffusion in capillaries within the same box body (see section 4). Polystyrene was used as the building material balancing the benefits and drawbacks of different materials for optical quality, chemical stability, physical stability and price. A plastic rack is available for the storage of the GCB boxes upside (see Figure 1a).

3. Counter-diffusion in the GCB

The GCB is designed mainly for counter-diffusion crystal growth experiments inside glass capillaries. Performing a counter-diffusion protein crystal growth experiment with the GCB is very easy using the so-called Gel Acupuncture MEthod (GAME) from which the GCB inherits many of its advantages, particularly the automatic search for optimum crystallisation conditions and the possibility of performing handling-free diffraction data collection.

The first step to implement this kind of experiment is to prepare the buffered sol of the gel precursor (Figure 2a). For simplicity we focus here only on agarose gels because they are reversible and have been demonstrated not to interact with protein molecules (Finet *et al*., 1998; Vidal *et al*., 1998). Other gels like silica or polyacrylamide can be used as well. Agarose gel preparation involves mixing the appropriate volume of buffer solution with agarose powder under continuous stirring for a final agarose concentration of 0.5 % w/v. This recommended agarose concentration is enough to maintain the capillaries and to avoid disordered fracture of the gel. The mixture is then heated to boiling in order to break the cross-links of the agarose fibres; at this point the agarose solution becomes transparent. After keeping the solution at this temperature for about two minutes under continuous stirring, pour 3 ml in the GCB box body (Figure 2c) in which the capillary holder was previously inserted (Figure 2b). The sol is then allowed to cool to room temperature, which takes few minutes. The cross-links of the agarose will be set to yield a gel with a strength that depends on the agarose type used in this study. We recommend an agarose with a gelling temperature of about 37ºC.

While the gel is setting, fill the capillaries with your buffered protein solution. To do this, cut the capillary to 7 cm length ensuring that the two ends of the capillary are open and introduce the lower end of the capillary into the protein solution (Figure 2d). The

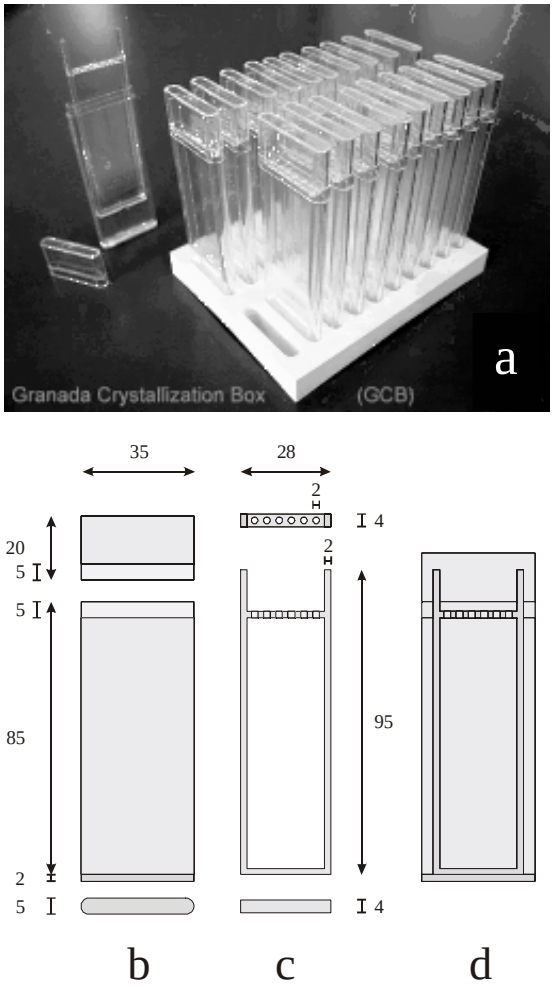

Figure 1

View of the Granada Crystallisation Box. b-d) Sketch of the GCB components showing: b) the body of the box, and c) the capillary holder. All dimensions are given in mm.

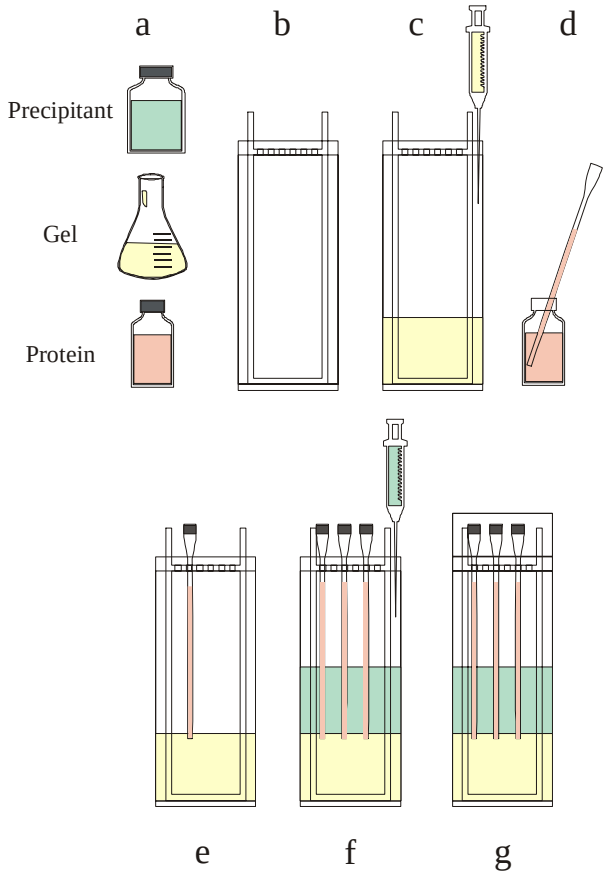

Figure 2

Schematic sequence of experimental set-up. See text for explanation.

solution enters the tube by capillarity. Fill the capillary to your convenience. Filling to a height of 5–6 cm is the common practice in our laboratory, because this length ensures the benefits of counter-diffusion for reasonable experimental times. Then seal the upper end of the capillary with a small amount of vacuum grease or your preferred sealing material.

Next step is to thrust the capillary into the gel layer (Figure 2e). To do it, introduce the capillary through one of the holes in the GCB capillary holder and insert it into the gel. Inserting the capillary 2–3 mm is just enough to keep it upside up, but note that this penetration length is a variable to tune the window of screened crystallisation conditions. Longest penetration length increases the physical buffer of the counter-diffusion arrangement lowering the rate at which the salt invades the capillary containing the protein. Up to six capillaries per GCB can be set in this way.

Finally, pour the buffered solution of your precipitating agent on top of the gel layer (Figure 2f). Typically a volume equal to the volume of the gel layer, i.e. 3 ml is recommended. Note that with this 1:1 ratio, the final precipitant concentration after homogenization of the experiment is half the initial concentration and close the box with the lid (Figure 2g) adding some vacuum grease to the body-lid joint before closing to completely avoid evaporation. Optionally, the box body and lid can be fixed covering the joint with adhesive tape for further stability while handling of the box.

Alternatively, another counter-diffusion setup can be implemented. The procedure is even simpler. Pour 3 ml of the buffered precipitating agent solution into the body of the GCB. Then, insert the capillary holder in the body of the GCB. Now, prepare an agarose sol of your buffered protein solution at an agarose concentration of 0.2 % w/v. According to our experience, this agarose concentration is the minimum to avoid the leakage from the capillary to the precipitating agent solution. To do it prepare the buffered agarose solution at a concentration of 2 % w/v and maintain the sol at 40ºC, i.e., just a few degrees above the gelling temperature. Pour your protein solution into an Eppendorf tube and hold it in your hand to maintain it at a temperature of 37ºC. Pour a volume of agarose sol equal to 1/10 of the volume of the protein solution into the Eppendorf and assure complete mixing by shaking the tube in your hand. Then fill and seal the capillary as above. Finally, introduce the capillary through one of the holes in the GCB capillary holder and soak its lower end into the solution of precipitating agent.

The amount of protein solution needed for these experiments depends obviously on the capillary diameter and on how much you fill the capillaries. Filling the capillaries up to a length of 50 or 60 mm is recommended in order to have a wide screening of the phase diagram. The recommended capillary diameter ranges from 0.1 mm for screening experiments to 0.5 mm for producing large crystals. Within these limits, the minimum protein solution volume required to implement GCB experiments is 0.39 µl per capillary for screening, the maximum is 11.78 µl for producing large crystals and around 4 µl for obtaining crystals of a suitable size for X-ray data collection.

4. Other methods

As GCB has been defined to implement counter-diffusion crystal growth inside capillaries, it is perfectly suited for experiments having a subset of the experimental requirements of counter-diffusion techniques. Many different experimental setups can be implemented by combining gelled or ungelled solutions with or without capillaries. Two evident examples are crystal growth inside capillaries by the batch method and inside gel by counter-diffusion directly in the body of the GCB. In the first case the benefits of counter-diffusion are not exploited and GCB is used just as a container providing a capillary holder, microscopy observation and easy handling; in the second case the benefits of capillaries are lost but those of counter-diffusion techniques are gained.

A capillary gel microbatch setup can be implemented easily by the following protocol: Prepare your buffered precipitating agent solution and mix it with agarose at 0.25 %. Boil it for 1 minute and then cool it to 40ºC. Maintain the sol at this temperature. Mix the appropriate volume of the protein solution with the appropriate volumes of the sol. In other words, proceed as to prepare a drop for microbatch. Then, suck the drop into the capillary by capillarity. Seal both ends of the capillary and hold it in the GCB capillary holder. The use of oil with microbatch technique inside capillaries can be also implemented (Moreno *et al*, 2002).

The growth of crystals inside gels by counter-diffusion is very simple. This method is ideal to prepare many large high-quality crystals for special purposes such as having many crystals for testing some experimental procedure, but it consumes a larger amount of protein than usual techniques. Make a sol of agarose at 1.2 % w/v by boiling the mixture for a couple of minutes. Prepare the protein solution. Let the agarose sol cool down to a temperature of about 40ºC. Keep the agarose sol at this temperature. Under stirring, mix one part of agarose sol with ten parts of protein solution, so that the final concentration of agarose is 0.12 % while that of protein is 9/10 of the initial one. This agarose concentration is the minimum to produce an actual gel network. Pour the mixture into the GCB and introduce the capillary holder. Allow the sol to cool to room temperature to set the gel. Once the gel is set, pour a solution of precipitating agent on top of the gelled protein solution layer. In this technique the capillary holder is not used to hold any capillary but to allow the easy extraction of the gel slice containing the crystals after the end of the growth. For instance, this implementation can be used to grow reinforced protein crystals inside silica gels (García-Ruiz *et al*, 1998). Prepare a tetramethoxysilane (TMOS) sol at the selected concentration (between 1 and 20 % w/v) by mixing your buffered protein solution with the appropriate amount of TMOS under continuous stirring. Pour 3 ml of the solution into the GCB body and introduce the capillary holder. Wait until the gel is set, which depends on pH and TMOS concentration. Then pour onto the gel the solution of the precipitating agent and close the GCB.

5. Previous experience

Many different proteins have been crystallized successfully using GCB up to now. These molecules show a broad range of molecular weight (from 5 to 1000 kDa), isoelectric point (from 3 to 12) and precipitant (salts, polymers, pH change). This information is summarized in Table I. Currently we are working in collaboration with different groups to expand this list on how crystallisation systems displaying very different behaviour can be implemented in GCB. Figure 3 shows several typical crystals grown in GCB from molecules listed in Table 1.

6. X ray measurements using GCB

Data collection at room temperature is made sensibly easier by using GCB as the crystals grow inside capillaries that can be X-ray capillaries. Therefore, no crystal handling or mounting is required; just take your capillary, seal the open end with your favourite sealing material after drying or cleaning a little bit the capillary end and mount the capillary on the goniometer head. If the range of displacement of your goniometer along the spindle axis is too short to accommodate the whole capillary, you can cut and seal it to get shorter capillary segments. In case the crystals are moving inside the

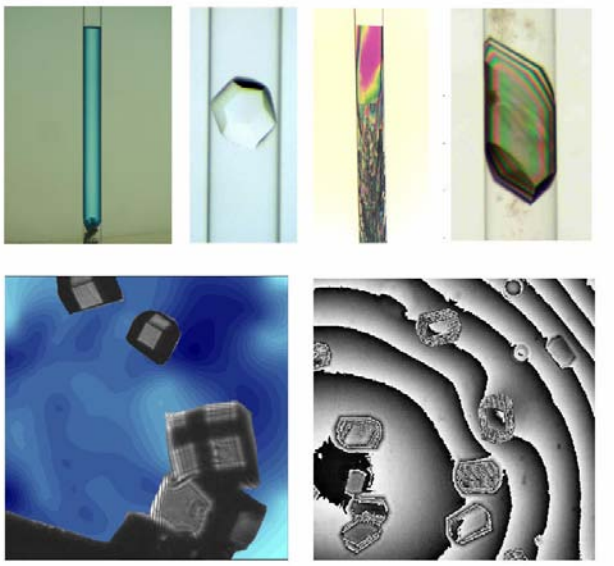

Figure 3

Some examples of proteins crystallised in the GCB. Top row from left to right: insulin, Glucose isomerase, Ferritin and Xylanase. Bottom row: phase interferometric images (Dubois & Novella, personal communication).

solution or you get too much scattering from the solution, you can extract part of the solution before sealing using cellulose fibers or any other absorber without touching the crystals.

Of particular interest for X-ray characterization is the possibility of producing shaped crystals. Far from the open end of the capillary, crystals nucleate at low supersaturation, and therefore at very low nucleation density, so the resulting crystals grow to large sizes and, eventually are large enough as to completely fill the capillary (Figure 3a). This produces cylindrical crystals that, in addition to making absorption correction trivial, are interesting for some X-ray techniques.

Cryocrystallography is possible within the capillaries used in the GCB (López-Jaramilllo *et al.*, 2001). The cryoprotectant can be included from the very beginning in the protein solution or added by diffusion using the same GCB setup before taking out the capillaries. If the protein solution in the capillary has been mixed with agarose, the crystals do not move. Their quality can be tested at home and full data collection at synchrotron facilities can be later performed on previously selected crystals. An all-in-one procedure to obtain crystals into capillaries with the incorporation of anomalous scattering halides in cryogenic solutions ready for flash cooling cryocrystallography has been demonstrated (Gavira *et al.*, 2002).

In special cases, when the X-ray technique to be used requires special handling, it is always possible to extract the crystals from the capillary and to handle the crystals in the way needed for the particular X-ray data collection setup.

7. Observation capabilities, transportation and use in microgravity

In spite of their suitability for the production of crystals for X-ray analysis, this function does not exhaust the capabilities of GCB. Experiments devoted to the observation of protein crystal growth are implemented easily and conveniently using the GCB. Both the box body and the capillaries are transparent, allowing a good quality microscopic imaging of the growing crystals (see Figure 3). For most microscopic observation setups, the thickness of the GCB inner space sets out of focus any feature in the walls of the box. Experiment handling is facilitated by the solid design of GCB:

Table 1 Crystals grown using the GCB.

Protein	MW (kDa)	pI	Precipitating system	pH	In collaboration with
Dehydroquinase	17.2	5.8	Ammoniun sulphate	7.4	Tibotec-Virco (Mechelen, Belgium)
Saicar synthase	35.5	5.3	Ammonium sulphate	7.5	Institute of Crystallography (Moscow, Russia)
Factor XIII	336	5.1	MES	7.0	Institut für Molekulare Biotechnologie (Jena, Germany)
Cytochrome C	15	4.6	Ammoniun sulphate	7.5	Instituto de Tecnologia Química e Biológica (Oeiras, Portugal)
Alliinase	51.5	6.5	Ammonium sulphate	8.2	Institut für Molekulare Biotechnologie (Jena, Germany)
Glucose Isomerase	173	3	Polyethylene glycol 8000	7.4	
Lumazine synthase	1000	5.4	Sodium potassium phosphate	8.7	Technical University Munich (Garching, Germany)
Lysozyme	14	11	Sodium chloride	4.5	
Thaumatin	22	12	Sodium potassium Tartrate	6.5	
Ferritin	456+PM_{Fe}	4.5	Cadmium sulphate	5.0	
Insulin	5	5.5	Sodium phosphate	9.0	
Concanavalin A	102	5.5	Polyethylene glycol 6000	8.0	
Catalase	250	5.6	Ammonium sulphate	5.2	Institute of Crystallography (Moscow, Russia)
Anti lysozyme camel antibody	27.9	8.4	Sodium formate	5.6	Vrije Universiteit (Brussel, Belgium)
Human liver FBPase	160	6.5	Polyethylene glycol 3350	9.0	
Apoferritin	456	4.3	Cadmium sulphate	5.0	
Xylanase	21	9.0	Polyethylene glycol 4000	7.0	
(Pro-Pro-Gly)10	7.6	5.2	Sodium acetate	5.4	University of Naples (Naples, Italy)
Triose Phosphate Isomerase	28.5	5.6	Ammonium sulphate	8.0	University of Liège (Sart Tilman, Belgium)

experiments can be handed from the storage shelf to the microscope and back without any risk. In addition, transport of crystals to synchrotron facilities can be performed using directly GCBs. A thermostated transportation box for GCB will be soon available.

Advanced observation techniques, like interferometry, can also be used on the GCB experiments inside or outside capillaries. Recently, holographic video and interferometry techniques have been successfully tested in capillaries mounted in GCB boxes (Figure 3 e-f) during the definition of a microgravity crystal growth facility (PROMISS) centred on interferometric observation of crystal growth (Zegers & Dubois, personal communication). In fact, GCB have been already used for microgravity experiments, as a passive, light and inexpensive facility for protein crystallisation. Details of the implementation and rationality of these experiments will be described elsewhere.

8. Further information

Up to date information on GCB and a collection of practical procedures and protocols are available from the LEC website (http://lec.ugr.es).

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